

IN THE SPECIFICATION

Please amend paragraphs [0008] through [0044] of the published version of the above-identified application, US 20060246092A1, as follows.

[0008] (a) removal of the intron out of the m2 gene  
SEQ ID NO: 59,

[0009] (b) introduction of a BclI site between the extracellular part and the transmembrane domain of the M2 protein,

[0010] (c) nucleotide (SEQ ID NO:9) and amino acid sequence (SEQ ID NO:10) of the extracellular part of the M2 protein of A/PR/8/34.

[0011] FIG. 2 Construction of pIPM2hB2Mm2s2.  
ori=origin of replication,

cat=chloramphenicol acetyltransferase,

bla= $\beta$ -lactamase,

lpp=lipoprotein, hB2M=human B<sub>2</sub>-microglobulin,

ompA-ss=signal sequence of the outer membrane protein A of *E. coli*,

ssDNA=single-stranded DNA,

M2e=extracellular part of the M2 protein.

[0012] (a) Construction flow scheme, SEQ ID NOS: 11 and 12 (FIG. 2a2), and SEQ ID NOS: 13, 14 and 15 (FIG. 2a3).

[0013] (b) Details of key sequences that include paired oligonucleotide and encoded amino acid sequences of SEQ ID NOS:44-51,

[0014] FIG. 3 Construction of pPLcIPM2HBcm.  
ori=origin of replication,

cat=chloramphenicol acetyltransferase,

bla= $\beta$ -lactamase,

HBC=hepatitis B core,

ssDNA=single-stranded DNA,

M2e=extracellular part of the M2 protein.

[0015] (a) Plasmid construction flow scheme including SEQ ID NO: 16 (FIG. 3a2) and SEQ ID NOS: 17 and 18 (FIG. 3a4),

[0016] (b) Sequence around the introduced BamHI restriction site in the hepatitis B core gene that includes paired oligonucleotide and encoded amino acid sequences of SEQ ID NOS:52-55 (FIG. 3b),

[0017] (c) Details of key sequences that include paired oligonucleotide and encoded amino acid sequences of SEQ ID NOS:56-61 (FIG. 3c).

[0018] FIG. 4 Analysis of the soluble fraction, corresponding to 150  $\mu$ l original culture, of strain MC1061[pcI857] containing the plasmids pPLc245 (control), pPLcA1 (expression of HBC) or pPLcIPM2HBcm (expression of IPM2HBcm) respectively, on a SDS 12.5% PAGE. After the electrophoresis the gel was stained with Coomassie brilliant blue.

MW=molecular weight marker,

NI=not induced culture,

I=induced culture.

[0019] FIG. 5 Analysis of the soluble fraction, corresponding to 150  $\mu$ l original culture, of strain MC1061[pcI857] transformed with pPLc245 (control), pPLcA1 (expression of HBC) or pPLcIPM2HBcm (expression of IPM2HBcm) respectively, as in FIG. 4. After electrophoresis, the relevant proteins were revealed by a Western blotting experiment. Detection with (A) a monoclonal antibody against HBC and (B) a

monoclonal antibody specific for the extracellular part of the M2 protein.

MW=molecular weight marker,

[0020] NI=not induced culture,

I=induced culture.

[0021] FIG. 6 Sequence of the amino terminus of the M2 protein compared to the amino terminus of IPM2HBcm as an oligonucleotide, its translated amino acid residue sequence (SEQ ID NOs: 19 and 20) the amino acid residue sequence of the amino terminus of the fusion protein IPM2HBcm (SEQ ID NO: 21), as experimentally determined. Sequence of A/Udorn/72 SEQ ID NO: 62, (Lamb and Zebedee, 1985).

[0022] FIG. 7 Soluble fractions of strain MC1061 [pcl857] transformed with pPLc245 (control), pPLcA1 (expression of HBc) or pPLcIPM2HBcm (expression of IPM2HBcm), respectively, analyzed in a native state by means of a dot blot. Detection with (A) a monoclonal antibody against HBc and (B) a monoclonal antibody specific for the extracellular part of the M2 protein. NI=not induced culture,

I=induced culture.

[0023] FIG. 8 Overview of (A1) rectal temperature, (A2) weight and (B) survival of the mice vaccinated with IPM2HBcm after a lethal challenge with 5 LD<sub>50</sub> m.a. A/PR/8/34. The statistical significance was calculated by the Fisher's exact test. Mice immunized with different doses of antigen were compared to the control group. The following results were obtained: for 50 µg IPM2HBcm p<0.001; for 10 µg p<0.005 and for the 5 µg dose p<0.05. FIG. 8C shows the survival of the mice vaccinated intraperitoneally with IPM2HBcm, and IM2HBcm, respectively, after a lethal challenge with 30 HAU X-47. FIG. 8D shows the survival of the mice vaccinated intranasally with

IPM2HBcm, and IM2HBcm, respectively, after a lethal challenge with HAU X-47.

[0024] FIG. 9 Analysis of the serum samples of the four set ups reported in FIG. 8. The pre-immune serum (a), the serum taken after the first (b), after the second (c) and after the third (d) immunization and the serum taken after challenge (e) were initially diluted 1/50. The consecutive dilution steps were 1/3. The plotted absorbance is a corrected value obtained as described in Results, Analysis of the serum samples.

[0025] FIG. 10 Construction of pPLcIM2HBcm including the site-directed mutagenesis mutator oligonucleotide (SEQ ID NO: 22) and selection oligonucleotide (SEQ ID NO: 23), ori=origin of replication, cat=chloramphenicol acetyltransferase, bla= $\beta$ -lactamase, M2e=extracellular part of the M2 protein, HBc=hepatitis B core.

[0026] FIG. 11 Analysis of the soluble fraction, containing 5  $\mu$ g HBc or I(P)M2HBcm (as determined in an ELISA (see Materials and Methods)), of strain MC1061 [pcI857] containing respectively the plasmids pPLc245 (control), pPLcAl (expression of HBc), pPLcIPM2HBcm (expression of the fusion protein IPM2HBcm with the extracellular part of the M2 protein derived from A/PR/8/34) or PPLcIM2HBcm (expression of IM2HBcm, containing the more universal M2 sequence) on a SDS 12.5% PAGE-gel. MW=molecular weight marker, NI=not induced, I=induced culture.

[0027] FIG. 12 Analysis of the soluble fraction, containing 2.5  $\mu$ g HBc or I(P)M2HBcm (as determined in an ELISA (see Materials and Methods)), of strain MC1061 [pcI857] containing respectively the plasmids pPLc245 (control), pPLcAl

(expression of HBc), pPLcIPM2HBcm (expression of IPM2HBcm) or pPLcIM2HBcm (expression of IM2HBcm) on a Western blot (see Materials and Methods). Detection with (A) a monoclonal antibody directed against HBc and (B) a monoclonal antibody specific for the extracellular part of the M2 protein.

MW=molecular weight marker,

NI=not induced,

I=induced culture.

[0028] FIG. 13 Overview of the oligonucleotides (SEQ ID NOs: 24-27) used for PCR amplification of HBc and i(p)m2HBc. `s` or `a` following the name of the oligonucleotide stands for the use of these primers in the sense (s) or anti-sense (a) orientation. The boxed sequence indicates the changed Leu codons.

[0029] FIG. 14 Overview of the construction of hbc and m2hbc fusions in vectors for *L. lactis*.

ori=origin of replication for *E. coli*,

ori(+)=origin of replication for *L. lactis*.

ermA and ermM=erythromycin resistance genes,

P1=*L. lactis* promoter,

bla=β-lactamase,

HBc=hepatitis B core,

M2e=extracellular part of the M2 protein,

usp45-ss=signal sequence of usp45,

mIL2=murine interleukin 2 and

mIL6=murine interleukin 6.

[0030] FIG. 15 Analysis of the expression of Hepatitis B core (HBc) and M2-HBc fusion proteins in a Western blot. An equivalent of  $10^9$  *L. lactis* bacteria of strain MG1363 containing respectively pTREX1 (control), pT1HBc, pT1HBcIL2, pT1HBcIL6 (expression of HBc alone or in combination with mIL2 or mIL6,

respectively), pT1PM2HBc, pT1PM2HBcIL2, pT1PM2HBcIL6 (expression of IPM2HBcm alone or in combination with mIL2 or mIL6, respectively), pT1M2HBc, pT1M2HBcIL2, pT1M2HBcIL6 (expression of IM2HBcm alone or in combination with mIL2 or mIL6, respectively), was analyzed in a SDS 12.5% PAGE-gel. The first antibody, p-anti-HBc (Dako Corporation, Carpinteria, Calif., USA) was diluted 5000 times. The bound antibodies were detected with a 1/2000 dilution of the polyclonal anti-rabbit IgG labeled with alkaline phosphatase (Southern Biotechnology Associates, Birmingham, Ala., USA). I(P)M2HBc stands for either IPM2HBcm or IM2HBcm.

MW=molecular weight marker,

C=control and

--expression of the antigen alone.

[0031] FIG. 16 Analysis of the expression of M2-HBc fusion proteins in a Western blot. An equivalent of 2 to  $3 \times 10^9$  *L. lactis* bacteria of strain MG1363 containing respectively pT1HBc (control), pT1PM2HBc, pT1PM2LHBC (expression of IPM2HBcm), pT1M2HBc, pT1M2LHBC (expression of IM2HBcm), was separated on a SDS 12.5% PAGE-gel. The fusion proteins were detected with an IgG fraction of a polyclonal mouse anti-M2e antibody (see Materials and Methods). The bound antibodies were detected with a 1/2000 dilution of the alkaline phosphatase conjugated polyclonal anti-mouse IgG ( $\gamma$ -chain specific) (Southern Biotechnology Associates, Birmingham, Ala., USA).

MW=molecular weight marker,

C=control,

E=leucine codons optimal for use in *E. coli*, and

L=leucine codons optimal for use in *L. lactis*. These are the plasmids pT1PM2LHBC and pT1M2LHBC, respectively. I(P)M2HBc stands for either IPM2HBcm or IM2HBcm.

[0032] FIG. 17 Overview of the oligonucleotides (SEQ ID NOS: 28-31) used for PCR amplification of the extracellular part of the M2 protein and C3d. `s` or `a` following the code name of the oligonucleotide stands for the use of these primers in the sense (s) or anti-sense (a) orientation. The boxed region indicates the changed Leu codons.

[0033] FIG. 18 Overview of the construction of m2c3d3 fusions in *L. lactis*.

ori=origin of replication for *E. coli*.

ori(+) = origin of replication for *L. lactis*,

ermA and ermM=erythromycin resistance genes,

P1=*L. lactis* promoter,

bla=β-lactamase,

M2e=extracellular part of the M2 protein,

usp45-ss=signal sequence of usp45,

spaX=anchor sequence derived from *Staphylococcus aureus* protein A,

C3d=complement protein 3 fragment d, and

mIL6=murine interleukin 6.

[0034] FIG. 19 Overview of the oligonucleotides (SEQ ID NOS: 32-34) used for PCR amplification of TTFC and m2TTFC.

`s` or `a` following the name of the oligonucleotide stands for the use of these primers in the sense (s) or anti-sense (a) orientation. The boxed region indicates the changed Leu codons.

[0035] FIG. 20 Overview of the construction of m2TTFC in vectors for *L. lactis*.

ori=origin of replication for *E. coli*,

ori(+) = origin of replication for *L. lactis*,

ermM and erm.mu.=erythromycin resistance genes,

P1=*L. lactis* promoter,

bla=β-lactamase,

TTFC=tetanus toxin fragment C,  
M2e=extracellular part of the M2 protein,  
usp45-ss=signal sequence of usp45,  
mIL2=murine interleukin 2, and  
mIL6=murine interleukin 6.

[0036] FIG. 21 Analysis of the expression of IPM2TTFC fusion protein in a Western blot. n equivalent of  $10^9$  *L. lactis* bacteria of strain MG1363 containing respectively pT1TT (control), pT1PM2LTT (expression of IPM2TT), pT1PM2LTTIL2 (expression of IPM2TT in combination with mIL2) or pT1PM2LTTIL6 (expression of IPM2TT in combination with mIL6), was analyzed in a SDS 10% PAGE-gel. The first antibody, an IgG fraction of a polyclonal mouse anti-M2e antibody (see Materials and Methods) was diluted 2500 times. The bound antibodies were detected with a 1/2000 dilution of the polyclonal anti-mouse IgG labeled with horseradish peroxidase (Southern Biotechnology Associates, Birmingham, Ala., USA). 30 mg 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo., USA), was dissolved in 10 ml methanol.) Afterwards 40 ml PBS, pH 7.4 and 150  $\mu$ l H<sub>2</sub>O<sub>2</sub> was added.

MW=molecular weight marker,  
-=expression of the antigen alone,  
mIL2=expression of the antigen in combination with mIL2,  
mIL6=expression of the antigen in combination with mIL6.

[0037] FIG. 22 Primer set (SEQ ID NOS: 35 and 36) used for PCR amplification of the secretion signal of the gp67 baculovirus protein.

[0038] FIG. 23 Primer set (SEQ ID NOS: 37 and 38) used for PCR amplification of the extracellular part of the M2 protein during construction of the sgpM2C3d3 fusion.

[0039] FIG. 24 Construction of the baculovirus transfer vector pACsgpM2C3d3.

bla=β-lactamase,

bold grey line=baculovirus homology region,

C3d=complement protein 3 fragment d,

M2e=extracellular part of the M2 protein (SEQ ID NO: 64),

ori=origin of replication,

phP=baculovirus polyhedrin promoter, and

sgp67=secretion signal of the gp67 baculovirus protein.

[0040] FIG. 25 Detail of nucleotide and amino acid key sequences of the sgpM2C3d3 fusion.

C3d=complement protein 3 fragment d (SEQ ID NOS: 68 and 67),

M2e=extracellular part of the M2 protein (SEQ ID NOS: 66 and 65), and

sgp67=secretion signal of the gp67 baculovirus protein.

[0041] FIG. 26 Analysis of recombinant AcNPV/sgpM2C3d3 baculovirus by PCR amplification of the polyhedrin locus (primers TTTACTGTTTCGTAACAGTTTG (SEQ ID NO. 4) and CAACAAACGCACAGAACATCTAG (SEQ ID NO. 5)). Control reactions were performed with the parental transfer vector pACsgpM2C3d3 and with wild type AcNPV baculovirus.

M=DNA size markers.

[0042] FIG. 27: Expression of secreted M2C3d3 by Sf9 insect cells infected with recombinant AcNPV/sgpM2C3d3 baculovirus as demonstrated by Western analysis (10% PAGE-gel) of harvested supernatant. Supernatant from mock infected cells or obtained after infection with wild type AcNPV baculovirus are included as a control.

MW=molecular weight markers.

[0043] FIG. 28 Overview of the survival of mice after a lethal challenge with 5 LD<sub>50</sub> m.a. X47. Mice vaccinated with 3X10 µg IM2HBCm are compared with passively immunized mice (P).

[0044] FIG. 29 Overview of the DNA vaccination constructs including the oligonucleotides of SEQ ID NOS: 39-43, RT=reverse transcriptase

PCMV=cytomegalovirus promoter

bla=β-lactamase

npt=neomycin resistance.